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Table of Contents

Page Number

| | |
|------------------------------|-----|
| Front cover | 1 |
| Standard Form SF298 | 2 |
| Table of Contents | 3 |
| Introduction | 4 |
| Body | 4-8 |
| Key Research Accomplishments | 8 |
| Reportable Outcomes | 8 |
| References | 9 |
| Conclusions | 9 |
| Appendix | |
| Reprints (2) | |
| Abstracts (1) | |
| Figures (9) | |

Introduction

HER2 overexpression is a poor prognostic indicator in breast cancer. HER2 amplification is associated with early tumor dissemination, rapid tumor progression, and increased invasiveness, implying that HER2 has a significant role in the metastatic phenotype. We have demonstrated that two key steps in the metastatic mechanism, angiogenesis and transendothelial migration, are augmented by HER2 expression, and we have linked Angiopoietin-2, a vascular destabilizing protein, to expression of HER2. The **objective** of this research is to determine if the metastatic advantage of HER2 expressing cancer cells is imparted by Angiopoietin-2 production, and further to determine if overexpression of HER2 is linked to Angiopoietin-2 expression. The **scope** of this research begins with two assays to test angiogenesis and endothelial cell retraction, a key step in transendothelial migration. Using several strategies, the research protocol tests tumor cell production of Angiopoietin-2 or blockade of Angiopoietin-2 to determine if Angiopoietin-2 modulates the metastatic steps in question. Further, breast cancer specimens are tested for concurrent expression of HER2 and Angiopoietin-2, and also correlated with stage and grade of the tumor. In addition, concurrent expression of related receptors (Epidermal Growth Factor receptor, HER3, and HER4) are also tested for correlation of Angiopoietin-2 expression.

Body

Specific aim #1 calls for testing the effect of manipulating Angiopoietin-2 production in MCF-7 breast cancer cells on in vitro models of key metastatic steps. The first series of experiments tested tumor cell induced endothelial cell retraction, a key step in tumor cell transendothelial migration. Angiopoietin-2 expression was manipulated in these cells using 3 techniques: 1) Stimulating or blocking HER2 signaling using Herceptin (a monoclonal antibody that binds to HER2 and prevents dimerization and signaling) or Heregulin $\beta 1$ (a ligand for HER3 and HER4, which upon binding induces dimerization with HER2 and subsequent signaling); 2) Direct application of Angiopoietin-2 into the assay or sequestration of tumor produced Angiopoietin-2 in the assay by treatment with soluble Tie-2/Fc receptor fusion protein; and 3) Angiopoietin-2 cDNA transfection in sense and antisense orientation to increase or decrease tumor cell production of Angiopoietin-2.

Experiment #1. Tumor cell induced endothelial cell retraction. MCF-7 cells or transfected MCF-7 cells (HER cells) that overexpress HER2 were cocultured for 3 hours onto intact, 5-day-old monolayers of human endothelial cells. We have shown that the HER2 overexpressing cells produce more Angiopoietin-2 than the parent cells (1). Figure 1 shows that HER2 overexpressing cells induce a greater degree of endothelial cell retraction as determined by A) number of tumor cells associated with endothelial cell retraction events, and B) the percent of subendothelial matrix that is exposed by retracting endothelial cells. The number of tumor cells associated with retraction events was determined by hand counting using SEM images. The matrix method was determined by capturing a digital image and calculating the percent of

subendothelial matrix exposed by retracting endothelial cells using image analysis software (SigmaScan Pro). $P < 0.001$ between groups using Student's t-test.

Experiment #2. HER2 signaling-induced endothelial cell retraction. MCF-7 or HER cells were pretreated with Herceptin or Heregulin $\beta 1$ to block or stimulate HER2 signaling. We have shown that HER2 signaling regulates Angiopoietin-2 production in MCF-7 cells (Figure 2). The MCF-7 cells were then cocultured for 12 hours onto the intact human endothelial cells. Image analysis software was used to determine the percent of subendothelial matrix exposed by retracting endothelial cells. Figure 3 shows enhanced phase contrast images of intact endothelial monolayers (A) after 12 hours of coculture, in which heregulin $\beta 1$ treatment (D) greatly increased the degree of endothelial retraction induced by coculture with MCF-7 cells (B). Conversely, endothelial cell retraction was effectively arrested by tumor cell treatment with Herceptin to block HER2 signaling (C). Figure 4 shows the degree of subendothelial matrix exposed, comparing control tumor cells (both MCF-7 and HER) with cells pretreated with heregulin $\beta 1$ or Herceptin. In both cell lines, statistically significant modulation of endothelial cell retraction was seen with manipulation of HER2 signaling, concurrent with modulation of Angiopoietin-2 production (see ref. 1, $p < 0.01$ Herceptin vs MCF-7; $p < 0.001$ Herceptin vs HER; $p < 0.05$ Heregulin $\beta 1$ vs tumor cells). Statistical analysis determined by Student's t-test between groups.

Experiment #3. Angiopoietin-2 induction of endothelial cell retraction. In this experiment, Angiopoietin-2 was directly applied in escalating doses to the intact endothelial monolayers in the retraction assay. Further, MCF-7 cells were pretreated with soluble Tie2/Fc receptor fusion protein (sTie2/Fc) to bind and sequester tumor cell released Angiopoietin-2, which may prevent Angiopoietin-2 stimulation of Tie-2 receptors on the endothelium. Figure 5 presents the percent of subendothelial matrix exposed upon endothelial cell retraction during exposure to increasing doses of Angiopoietin-2, and compares the retraction response with MCF-7 and HER cell induced retraction. Low doses of Angiopoietin-2 failed to induce significant retraction, but at 200 ng/ml, Angiopoietin-2 induced a great degree of retraction, on scale with HER cells ($p < 0.01$ vs MCF-7). (Low dose failure may be attributable to failure to overcome intrinsic Angiopoietin-1 stimulation of the Tie-2 receptor, to which it binds with equal affinity. The role of Angiopoietin-1 is endothelial stabilization, an opposite effect of Angiopoietin-2.) This experiment suggests that Angiopoietin-2 is likely a factor in tumor cell induced endothelial cell retraction. Figure 6 presents the percent of subendothelial matrix exposed upon endothelial cell retraction during coculture with untreated MCF-7 cells or MCF-7 cells treated with increasing doses of sTie2/Fc. The sequestration of Angiopoietin-2 significantly altered the ability of MCF-7 cells to induce endothelial cell retraction at a dose of 200 ng/ml sTie2/Fc ($p < 0.05$ vs MCF-7). This experiment supports the results of the direct Angiopoietin-2 application experiments. Statistical analysis determined by Student's t-test between groups.

Experiment #4. This experiment is designed to confirm the role of tumor produced Angiopoietin-2 in the metastatic step of transendothelial migration of tumor cells. This experiment calls for transfection of the full-length cDNA into MCF-7 cells to induce promotable Angiopoietin-2 expression. The full-length cDNA has been obtained, and successfully placed in the PCDNA 3.1 vector. Transfection strategies are currently being implemented. Additionally,

this experimental protocol calls for transfection of antisense cDNA strands into the MCF-7 cells in an effort to prevent cell translation of the Angiopoietin-2 protein. Five antisense clones have been obtained to date. These appear to have operable antisense function by screening RT-PCR for intact Angiopoietin-2 mRNA. Further testing with Northern and Western blot is ongoing. Once these transfected cells are experimentally operational, we will further test the role of Angiopoietin-2 to induce endothelial cell retraction.

Experiment #5. The next series of experiments evaluated the role of tumor produced Angiopoietin-2 in the mechanism of angioinvasion through microvessel dismantling. Angioinvasion was studied using a 3 dimensional in vitro microvessel dismantling assay of isolated rat microvessels embedded in collagen I gels (for full protocol please see ref. 2, reprint included in the appendix). Figure 1 (in reprint of publication "HER2 signaling-induced microvessel dismantling") demonstrates dismantling of embedded microvessels upon exposure to HER2 overexpressing MCF-7 cells (HER). After 12 hours of coculture with HER cells, embedded microvessels demonstrate areas of discontinuity, with architectural dismantling. Calculating the length of intact microvessels, and comparing to length of control, untreated microvessels quantitated the effect. We compared MCF-7 cells with HER cells, which express significantly more Angiopoietin-2 (1). MCF-7 or HER cells were cocultured with intact microvessels in a 3 dimensional matrix. After 12 hours, the microvessels were stained with Gs-1 lectin, specific for endothelium. The microvessels were imaged, and the cumulative length of neomicrovessels was calculated and compared to untreated microvessels. Figure 2 (reprint) shows that exposure to the tumor cells induce a time dependent dismantling of microvessels, with HER cells inducing a significantly more rapid and more extensive effect ($p < 0.05$ vs MCF-7). To further implicate HER2 signaling as a mechanism for this metastatic step, we pretreated MCF-7 cells with Herceptin or Heregulin $\beta 1$ to block or induce HER2 signaling as described in our grant protocol. Figures 3 and 4 (reprint) demonstrate that blockade or stimulation of HER2 signaling can dose dependently limit or enhance tumor cell induced microvessel dismantling ($p < 0.01$). We further demonstrate in Figure 5 (reprint) that other HER2 expressing breast cancer cell lines can induce microvessel dismantling, and in at least one additional line the effect is blocked by Herceptin blockade of HER2 signaling.

Experiment #6. In this experiment (also included in reprint publication, "HER2 signaling-induced microvessel dismantling") we tested the direct application of Angiopoietin-2 protein to induce microvessel dismantling. Further, we used sTie2/Fc to sequester tumor produced Angiopoietin-2. Microvessels in the dismantling assay were exposed to a crude prep of Angiopoietin-2 protein in increasing doses up to 200 ng/ml. No significant induction of microvessel dismantling was identified. (Subsequent to this publication, a commercially available, purified protein was obtained. This was used in the Angiopoietin-2 protein experiment ((#3)) described above. These experiments will be repeated with the new protein.) We also treated the MCF-7 cells with sTie2/Fc to sequester Angiopoietin-2, and exposed the microvessels to these pretreated cells. Figure 6 (in reprint) shows that sTie2/Fc dose-dependently-inhibited induced dismantling, reaching significance at 200 ng/ml ($p < 0.01$.) Further experiments described in the Statement of Work using molecular modifications of the tumor cells to enhance

or limit the tumor cell production of Angiopoietin-2 (see Experiment # 4) are planned when the modified tumor cells are available.

Experiment #7. These experiments were not described in the Statement of Work, but were designed to further elucidate the mechanism of HER2 signaling and Angiopoietin-2 induction of endothelial cell retraction. We postulated that endothelial cells retract after the binding of Angiopoietin-2 to the Tie2 receptor on the endothelial cell induces the dissociation of the catenin proteins from vascular endothelial (VE) cadherin. These proteins are key structural proteins of the adherens junctions of the endothelium. Under appropriate stimulation, a sequential dissociation of γ , β , and then α catenin from VE cadherin breaks the adherens junction link to the cytoskeleton, resulting in retraction and rounding of the endothelial cell. In these experiments, we tested intact human endothelial cell monolayers for dissociation of the catenins from VE cadherin after exposure to MCF-7, and further tested the MCF-7 cells after treatment with Herceptin and Heregulin $\beta 1$ to manipulate HER2 signaling in these cells. Using immunoprecipitation of VE cadherin after exposure of the monolayer to tumor cells, we determined the quantity of the catenins, which remained associated with VE cadherin by Western blot analysis. Figure 7 shows the quantity of the catenins linked to VE cadherin over time of exposure to MCF-7 cells. Western blots were digitized and the densitometric intensity was determined and compared to control. The data is reported as percent of control (untreated) monolayers. A time dependent loss of associated catenins is clearly demonstrated, with greater than 90% loss of γ catenin seen at 24 hrs ($p < 0.01$). Further, HER2 signaling regulation using Herceptin and Heregulin $\beta 1$ significantly altered the dissociation curve (Figure 8). A 50% reduction in γ catenin dissociation was seen at 24 hrs after treatment with Herceptin. Heregulin $\beta 1$ significantly augmented MCF-7 induced γ catenin dissociation ($p < 0.05$), achieving equivalence with the result of HER cell induction of catenin dissociation. (Recall that HER2 signaling modulations alters the tumor cell production of Angiopoietin-2). These results imply that the mechanism of HER2 signaling induced endothelial cell retraction likely includes dissociation of the adherens junction proteins, with loss of continuity with the cytoskeleton.

Experiment #8. This experiment tested Angiopoietin-2 sequestration with sTie2/Fc from MCF-7 cells in coculture with endothelial monolayers. After pretreatment with increasing doses of Tie2/Fc, MCF-7 cells were cocultured with endothelial monolayers. After cell lysis, the lysate was immunoprecipitated with anti-VE cadherin antibody. Western blot analysis of the immunoprecipitate for γ catenin was performed. Figure 9 shows that sequestration of Angiopoietin-2 with sTie2/Fc significantly reduced the γ catenin dissociation induced by MCF-7 cells back to 50% of control ($p < 0.05$ vs MCF-7), similar to Herceptin treatment. This experiment further implicates tumor cell produced Angiopoietin-2 as part of the mechanism of the HER2 signaling induced, metastatic phenotype. Ongoing studies are testing the direct application of Angiopoietin-2 protein to induce γ catenin dissociation. Further, MCF-7 cells engineered to modify the production of Angiopoietin-2 will also be tested in this model.

Specific Aim #2 calls for the determination of concurrent expression of HER2 and Angiopoietin-2 in breast cancer specimens with the aim to determine if HER2 expression is

linked to Angiopoietin-2 expression in breast cancer. The methods will use laser capture microdissection as described in the proposal protocols. Because HER2 is the signaling subunit of heterodimers with other types I growth factor receptors (EGFR, HER3, and HER4) we will also determine relative levels of these receptors in the breast specimens. To date we have collected over 50 cancer specimens, and have tested 11 cancers. We have also tested three normal breast specimens along with placenta as a positive control. Figure 10 illustrates these results. Of 11 breast cancers, 5 overexpress HER2 (expect approximately 25-30% of breast cancers to be positive for HER2 by historical literature). Seven cancers express Angiopoietin-2, included all of the HER2 expressing cancers. These early data support the HER2 signaling link to Angiopoietin-2 production, but also indicate that other signaling pathways are likely to also induce Angiopoietin-2 production. Specific primers for RT-PCR for the associated receptors are being developed and tested at this time. Further cancer specimen testing will begin when all the appropriate primers are available.

Key Research Accomplishments

- Determined that HER2 signaling induces a metastatic phenotype in breast cancer involving endothelial cell retraction (as a key step in transendothelial migration) and microvessel dismantling (as a potential avenue of angioinvasion.)
- Determined that Angiopoietin-2 can induce endothelial cell retraction, and is likely a key factor in the mechanism of the HER2 signaling induced metastatic phenotype
- Determined that the mechanism of endothelial cell retraction involves dissociation of the catenin proteins from VE cadherin, and loss of adherens junctional linkage to the cytoskeleton
- Determined that Angiopoietin-2 is likely involved in the mechanism of HER2 signaling induced microvessel dismantling, but is not the only factor in this mechanism.
- Identified that concurrent expression of HER2 and Angiopoietin-2 exists in breast cancer, although alternate signaling pathways are likely to also influence Angiopoietin-2 production in cancer cells.

Reportable Outcomes

1. Carter WB. HER2 signaling-induced microvessel dismantling. *Surgery* 2001;130:382-387.
2. Carter WB, Ward MD. HER2 signaling-induced microvessel dismantling. Abstract. Presented at the Society of University Surgeons, February 10, 2001, Chicago, IL.
3. Carter WB, Hoying JB, and Williams SK. HER2 overexpression enhances tumor cell transendothelial migration. Abstract. Presented at the Society of Surgical Oncology, March 12, 2001, Washington, D.C.

Conclusions

The work to date has substantially increased the knowledge available about the mechanisms involved in the development of a metastatic phenotype associated with HER2 overexpression. We have shown that at least two metastatic mechanistic pathways are enhanced by HER2 signaling; 1) endothelial cell retraction and transendothelial migration, and 2) microvessel dismantling as a portal for angiogenesis. Further, these metastatic pathways appear to involve Angiopoietin-2, a vascular destabilizing protein. The work presented identifies that the Angiopoietin-2/Tie-2 receptor pathway is likely a key intermediary step in the metastatic phenotype, and a worthy therapeutic target. Further, the determination of Angiopoietin-2 expression in breast cancer may suggest an appropriate tumor marker indicating greater metastatic or angiogenic potential. The remaining experiments designed in this study are likely to offer additional insights into these mechanisms, and perhaps elucidate other potential clinical targets or markers.

References

1. Carter WB, and Ward MD. HER2 regulatory control of angiopoietin-2 in breast cancer. *Surgery*. 2000;128:153-158.
2. Carter WB. HER2 signaling-induced microvessel dismantling. *Surgery* 2001;130:382-387.

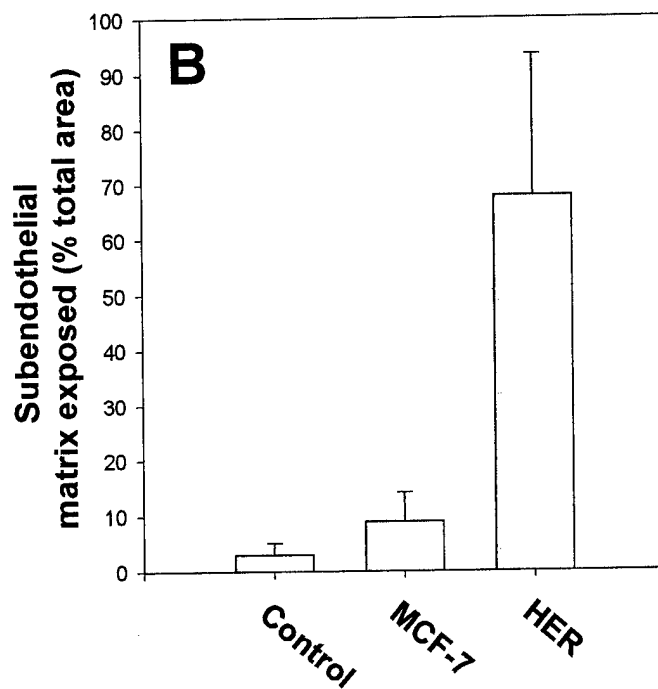
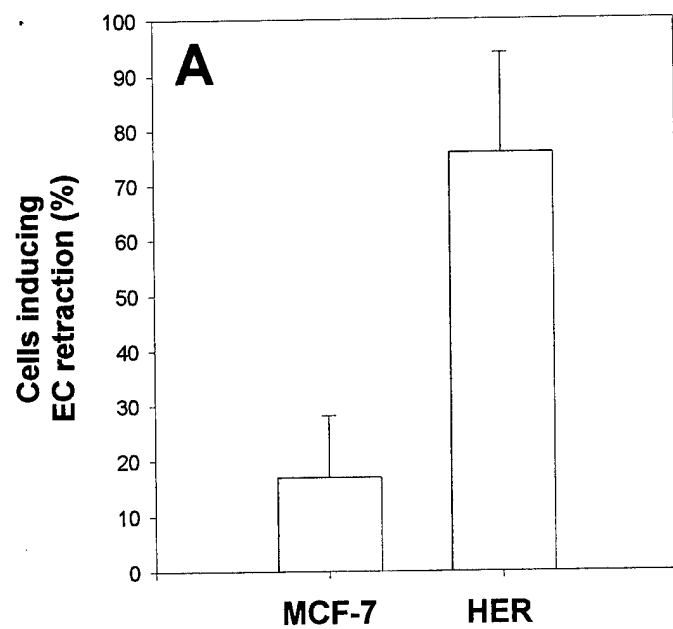


Figure 1

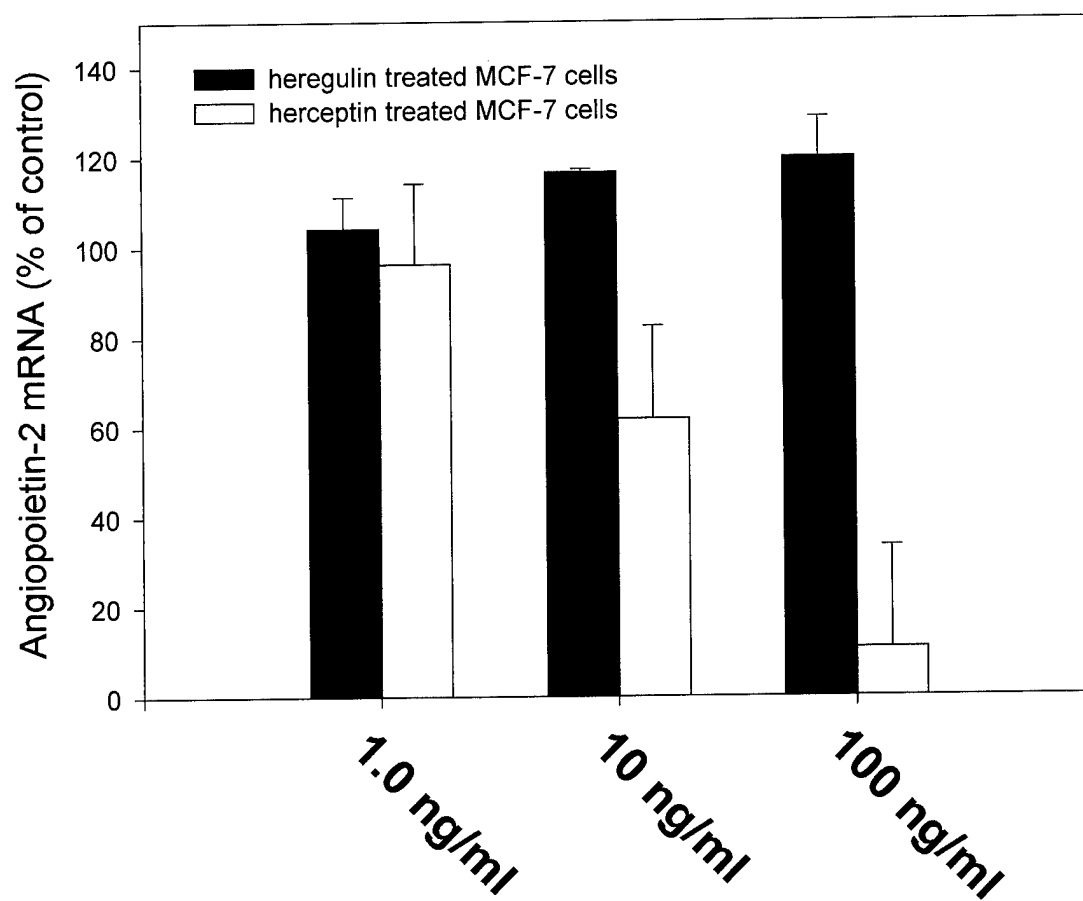


Figure 2

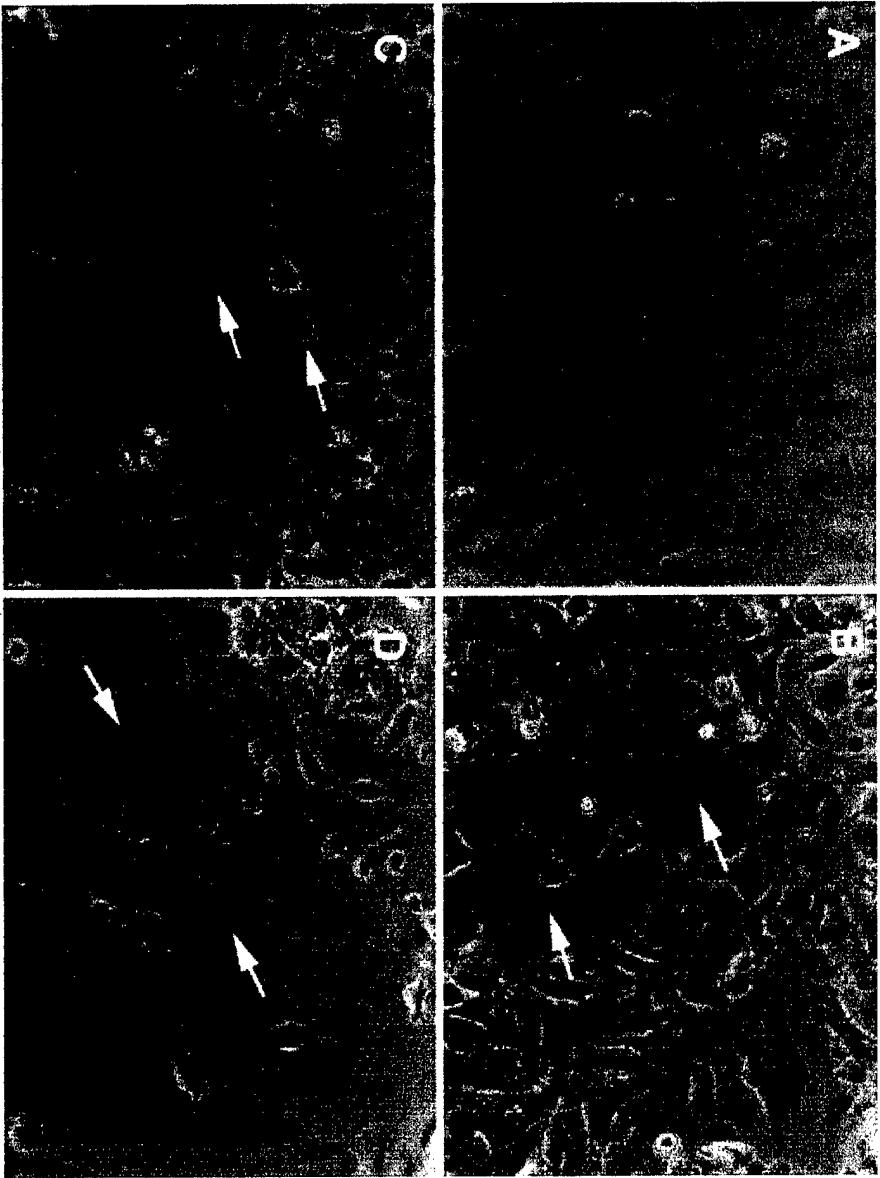


Figure 3

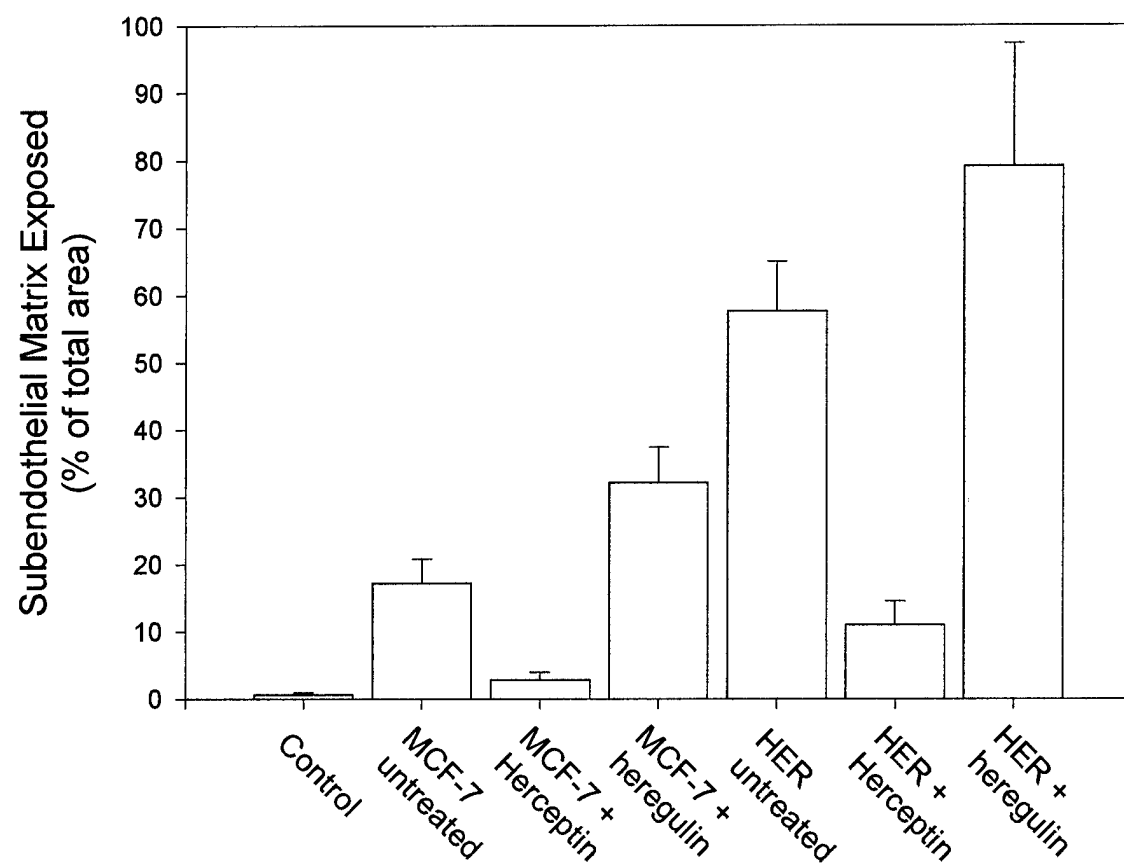


Figure 4

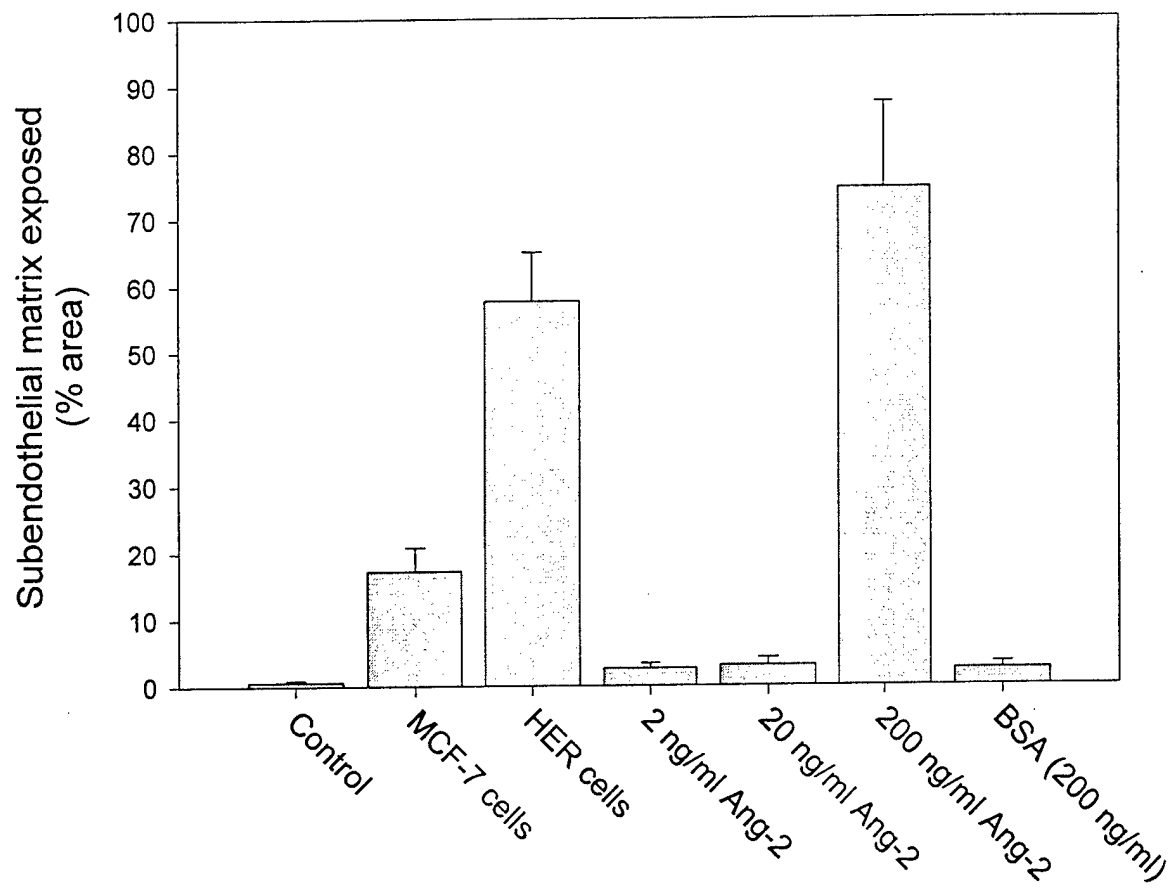


Figure 5

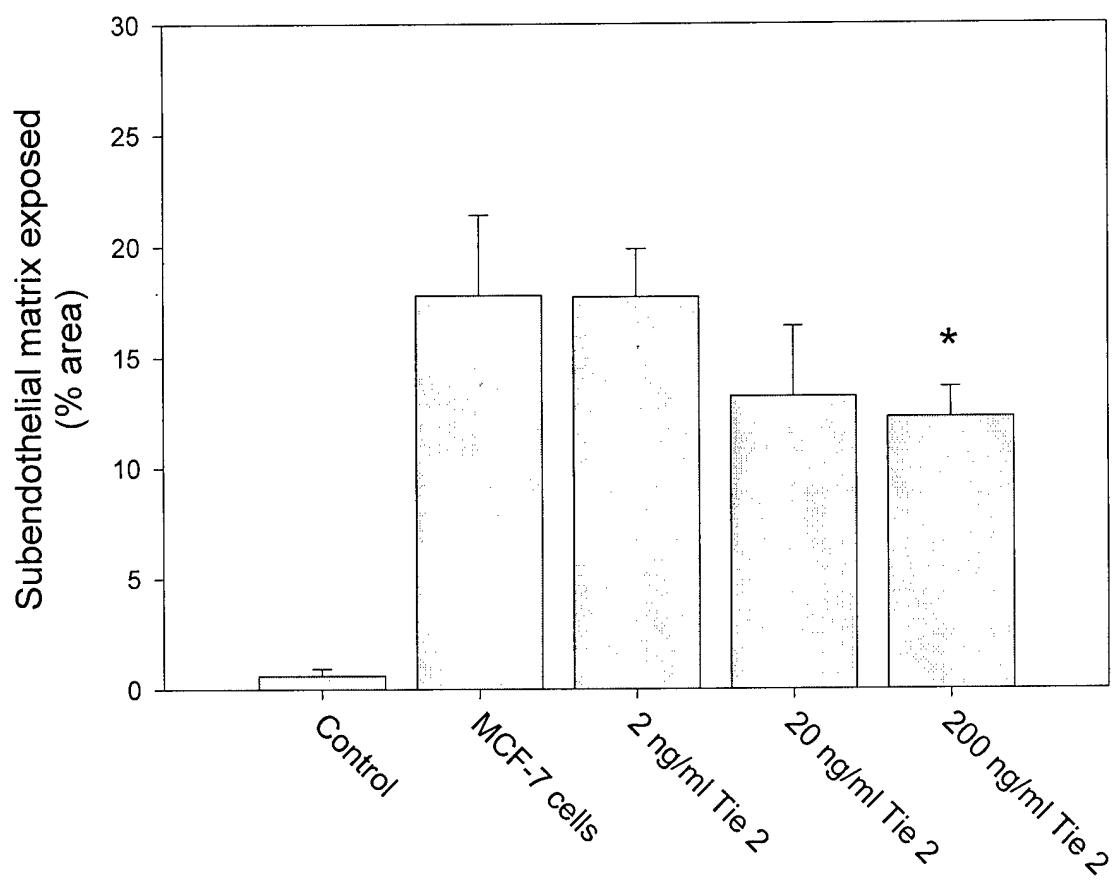


Figure 6

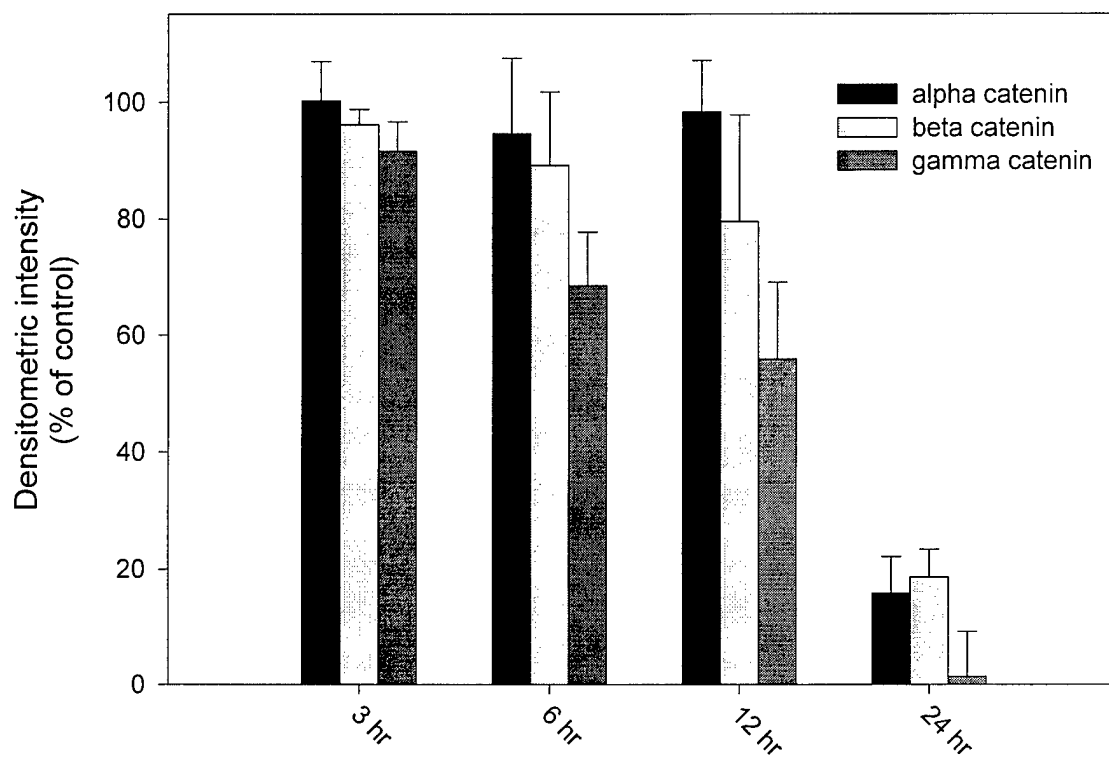


Figure 7

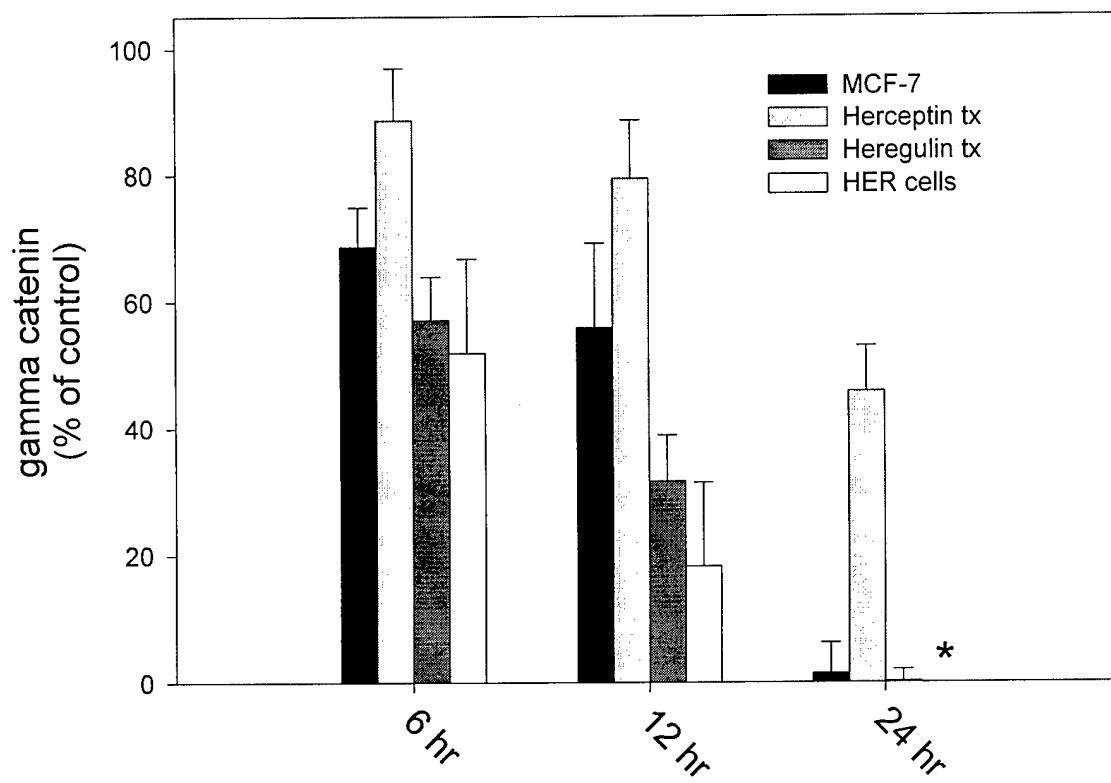


Figure 8

MCF-7
Control
sTie2 200
sTie2 500

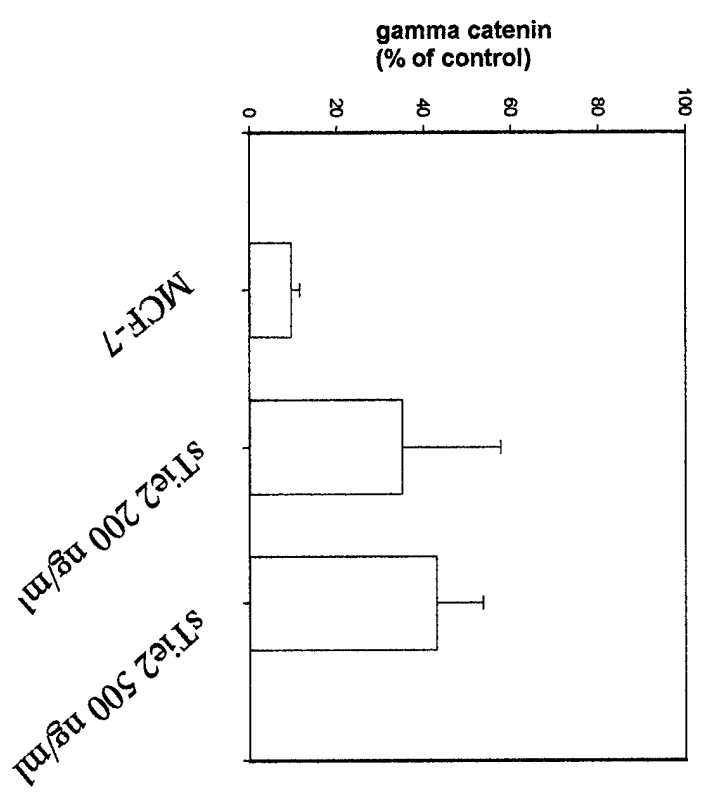


Figure 9

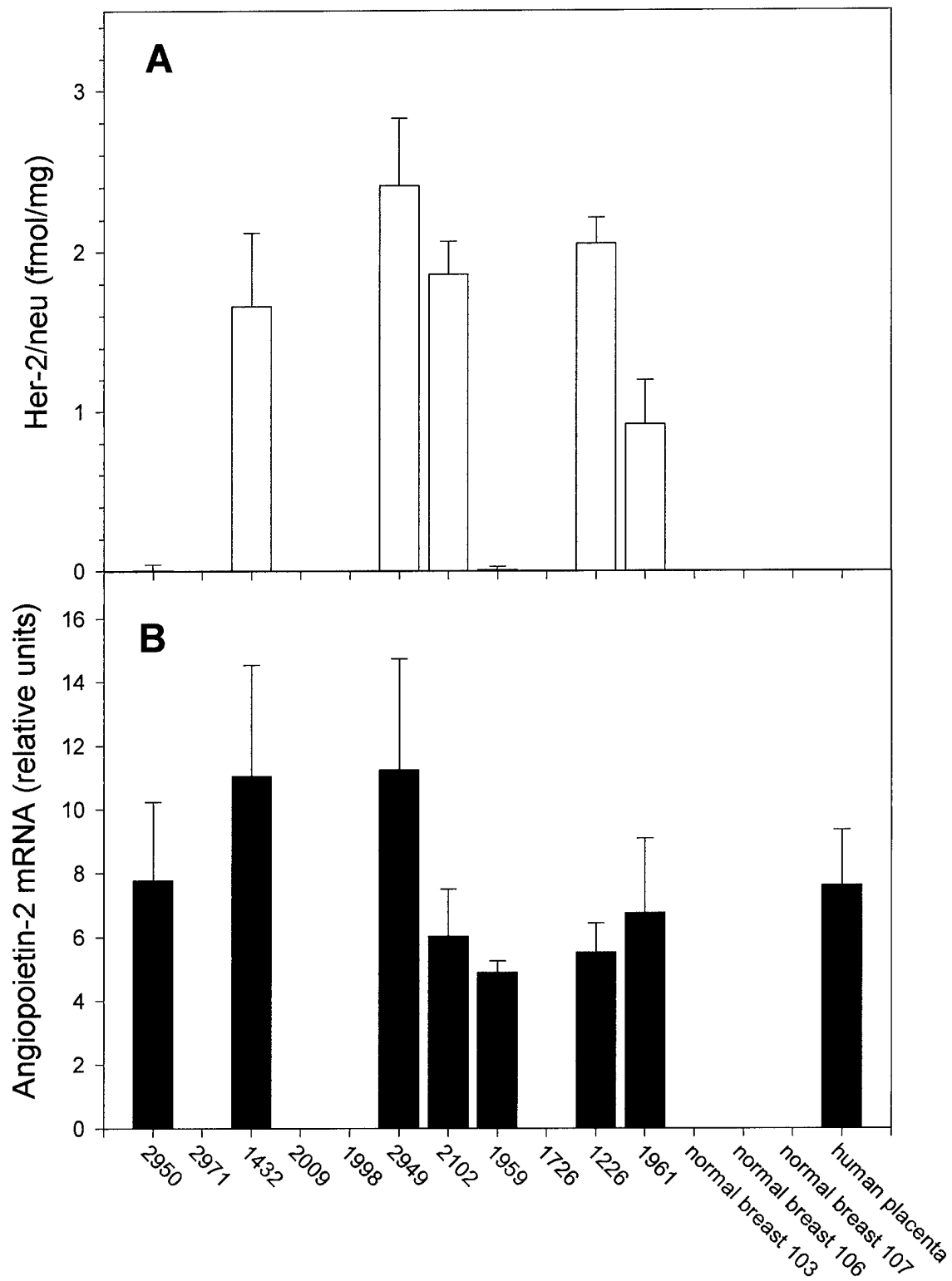


Figure 10

HER2 regulatory control of angiopoietin-2 in breast cancer

W. Bradford Carter, MD, and Michael D. Ward, BS, Norfolk, Va

Background. HER2 overexpression is a marker of aggressive breast cancer. Tumors that overexpress HER2 induce endothelial cell retraction and endothelial destabilization. Because angiopoietin-2 (Ang-2) also destabilizes microvessels, we postulated that HER2 signaling upregulates Ang-2 as a mechanism of angiogenesis.

Methods. We tested human breast cancers and breast cancer cell lines for coexpression of HER2 and Ang-2 with Northern blot, reverse transcriptase-polymerase chain reaction, and enzyme-linked immunosorbent assay. Further, we manipulated HER2 signaling with 100 ng/mL MAbHu HER2 (Herceptin; Genentech, San Francisco, Calif) and Heregulin $\beta 1$ (100 ng/mL; R&D Systems, Inc, Minneapolis, Minn) to test for HER2 regulation of Ang-2 production.

Results. Three of 4 breast cancer cell lines expressed HER2 protein and Ang-2 mRNA. HER cells, a stably transfected cell line that overexpresses HER2 6-fold, showed a 430% increase in Ang-2 mRNA compared to parental MCF-7 cells. Heregulin $\beta 1$ stimulation of HER2 signaling in MCF-7 cells increased Ang-2 by 20% ($P < .05$). HER2 signaling blockade with 100 ng/mL Herceptin reduced Ang-2 mRNA 90% ($P < .001$). Five of 11 cancers expressed both HER2 and Ang-2; 2 cancers expressed only Ang-2.

Conclusions. We conclude that human breast cancers express Ang-2. HER2 signaling appears to regulate Ang-2 expression, although other signaling pathways may also regulate Ang-2. Ang-2 may be a therapeutic target in these cancers and may define which patients would benefit from Herceptin therapy. (*Surgery* 2000;128:153-8.)

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OVEREXPRESSION OF HER2 (neu, C-erb B2), a type I growth factor receptor, imparts an aggressive metastatic phenotype and poor prognosis in breast cancer. In fact, HER2 is the most powerful predictor of disease-free and overall survival after lymph node status.¹⁻³ HER2 expression in breast cancer cells is inversely related to the estrogen receptor.⁴ It is believed that as the cell loses estrogen receptor expression, the cellular functions are replaced by upregulation of the type I growth factor receptors, which are involved in growth, differentiation, and survival.^{5,6}

This family of tyrosine kinase receptors is expressed in a wide range of different cell types. Four distinct members have been identified: EGFR (C-erb B1), HER2 (C-erb B2, neu), HER3 (C-erbB3), and HER4 (C-erbB4). The ligands for these receptors are the neuregulins, which bind to HER3 or HER4, causing heterodimerization with HER2.^{7,8} HER2 is the signaling subunit and has no independent ligand. Antibody blockade of HER2, preventing heterodimerization, eliminates neuregulin-stimulated signaling.⁹⁻¹²

HER2 expression imparts a metastatic advantage to the cell. Blockade of HER2 dimerization and signaling delays return to a tumor growth phase and induces tumor regression in breast cancers that express HER2.^{13,14} To date, very little information has been published that establishes the metastatic mechanisms that are enhanced by the cellular expression of this receptor. We have postulated that the expression and signaling of HER2 is linked to cellular production of a factor that influences the metastatic phenotype. Recently, Oh et al¹⁵ have identified downstream alterations in gene expression as a direct consequence

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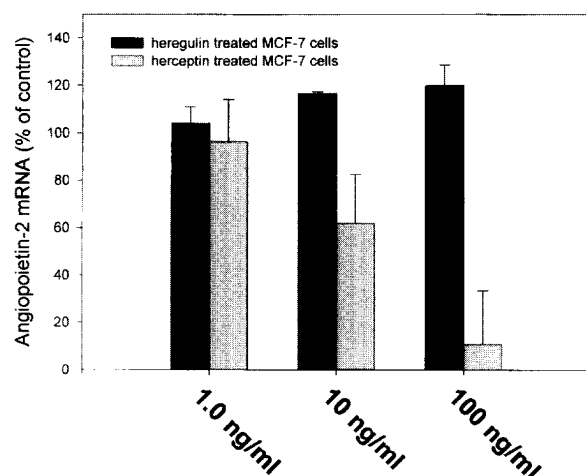


Fig 2. Dose response of Ang-2 mRNA expression to heregulin β 1 and Herceptin treatment. Total RNA (2 μ g) from MCF-7 cells treated with either Herceptin or Heregulin β 1 for 24 hours. Ang-2 mRNA was determined by semiquantitative RT-PCR. Significance was achieved at 100 ng/mL of Heregulin β 1 ($P < .05$) and 10 ng/mL Herceptin ($P < .01$). Product from RT-PCR was normalized to concurrent β -actin mRNA RT-PCR, and statistical analysis was performed after normalization was achieved as a percent of control.

and signaling. Herceptin blockade of HER2 signaling (100 ng/mL) reduced Ang-2 mRNA production (Fig 1). Conversely, stimulation of HER2 signaling with Heregulin β 1 (1.0 ng/mL) upregulated Ang-2 mRNA (Fig 1).

Using RT-PCR with specific primers for Ang-2, we tested pretreated MCF-7 cells for the production of Ang-2. Using 3 doses of Herceptin (1.0, 10, and 100 ng/mL) and Heregulin β 1 (1.0, 10, and 100 ng/mL), we constructed dose-response curves for Ang-2 production (Fig 2). The Herceptin-treated cell lines showed a marked decrease in Ang-2 mRNA production, determined by densitometry normalized to concurrent β -actin RT-PCR. A 90% reduction in Ang-2 mRNA was seen in MCF-7 cells treated with 100 ng/mL of Herceptin ($P < .001$ vs untreated cells). Pretreatment with Heregulin β 1 dose-dependently increased Ang-2 mRNA production (Fig 2). At 100 ng/mL of Heregulin β 1, there was a 20% increase in Ang-2 mRNA compared with control cells ($P < .05$).

To determine whether HER2 expression is linked to Ang-2 mRNA production in other breast cancer cell lines, we performed RT-PCR for Ang-2 using SK-BR3, MDA-MB-175, and MDA-MB-231 cell lines. We also tested HER cells, a stably transfected cell line derived from MCF-7 cells that overexpress HER2 6-fold. Fig 3 shows the relative expression of HER2 protein and Ang-2 mRNA in the cell lines.

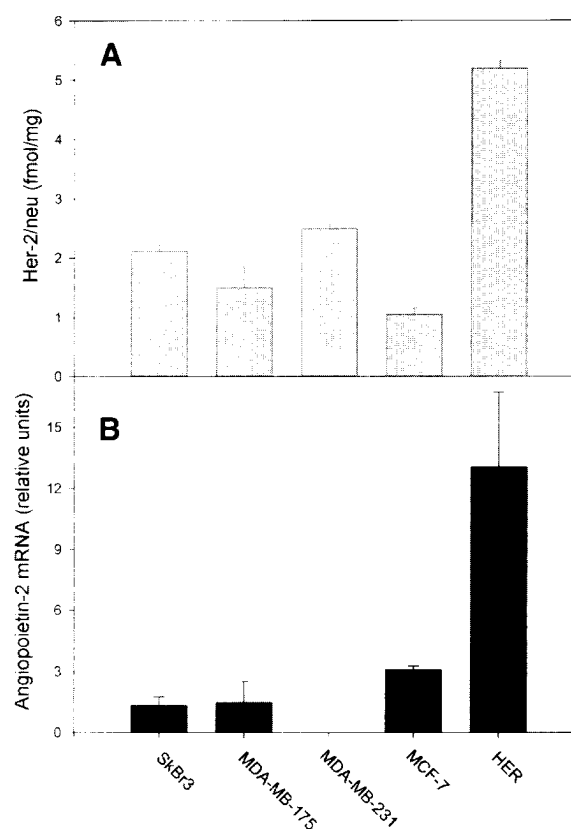


Fig 3. A. Expression of HER2 (femtomole/milligram) in breast cancer cell lines, determined by ELISA. **B.** Ang-2 expression in human breast cancer cell lines by RT-PCR with specific primers for Ang-2 (relative units determined by normalization to concurrent β -actin mRNA RT-PCR).

All of these cell lines expressed HER2 protein (Fig 3, A), and all but MDA-MB-231 expressed Ang-2 mRNA.

Lastly, we sought to determine whether Ang-2 mRNA production was found in human breast cancers that express HER2. Eleven archived breast cancer specimens and 3 normal breast epithelium specimens were assessed for HER2 protein by ELISA and for Ang-2 mRNA by RT-PCR. Five of 11 human breast cancers expressed HER2 (Fig 4, A), and 7 of 11 breast cancer tissues produced Ang-2 mRNA (Fig 4, B). All 5 HER2 expressors produced Ang-2 mRNA. There was no HER2 expression or Ang-2 mRNA detected in normal human breast epithelium.

DISCUSSION

HER2 overexpression is associated with a poor clinical prognosis in which patients have shorter disease-free and overall survival.¹⁻³ These findings suggest that HER2 expression is linked to downstream changes in gene expression, which enhance

the cells' metastatic ability. In this report, we sought to establish a link between HER2 expression and Ang-2 production, a putative vascular destabilizing factor. In previous work, we have determined that the enhanced metastatic phenotype of breast cancer cells that express HER2 appeared to be at least partly due to transendothelial migration of the tumor cells. The mechanism of transmigration appears to be the induction of EC retraction, with loss of EC-cell contact and barrier function. Because of the vascular destabilizing influence of cancer cells that are stimulated by HER2 signaling, we postulated that HER2 induces upregulation of Ang-2 with the profound effects on the endothelium noted. Blockade of HER2 signaling with the use of Herceptin reduced Ang-2 mRNA production by 90% in our study, although stimulation of HER2 signaling increased Ang-2 expression. These data suggest that HER2 signaling influences Ang-2 mRNA production, which supports our hypothesis. It is possible that Ang-2 production may be a mechanism of HER2-induced metastatic capability. Alternately, HER2 influence on cell proliferation or cell cycle regulation may be at least partly responsible for the alteration in Ang-2 expression. Further studies are necessary to demonstrate a direct effect of HER2 signaling on Ang-2 transcription and to identify which transcription factors may mediate the effect.

We have shown that MCF-7 cells, which express normal quantities of HER2 (1.0 fmol/mg tissue), produced Ang-2 mRNA. MCF-7 cells are considered to have low metastatic capability and in vitro induce a modest EC retraction response on endothelial monolayers (unpublished data). The MCF-7 transfected cell line (HER cells that overexpress HER2) show a much greater in vitro metastatic aggressiveness and a profound increase in Ang-2 mRNA. Antibody blockade of HER2 signaling has significantly reduced the aggressive phenotype of HER2 that expresses breast cancers both in vivo^{13,14} and in vitro and reflect the changes seen in Ang-2 expression in this study. Oh et al¹⁵ also identified several genes with expression levels that were altered in MCF-7 cells when modified by transfection to overexpress HER2. We further studied other breast cancer cell lines that overexpress HER2 to apply this hypothesis outside the MCF-7 model. We identified Ang-2 production in 2 of 3 of these cell lines. These studies indicate that HER2 signaling may influence alteration of downstream gene expression, specifically Ang-2.

The overexpression of HER2 is found in 25% to 30% of breast cancers. In the specimens tested for this study, 5 of 11 cancers expressed HER2, and all

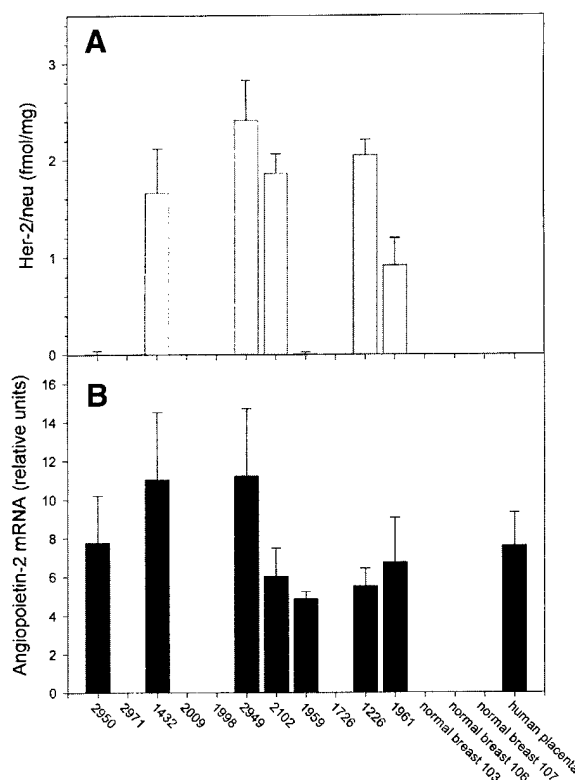


Fig 4. A, Expression of HER2 (femtomole/milligram) in human breast cancer specimens, determined by ELISA. **B,** Ang-2 expression in human breast cancer specimens and normal breast epithelium by RT-PCR with specific primers for Ang-2 (relative units determined by normalization to concurrent β -actin mRNA RT-PCR). Human placenta RNA was used as a positive control for Ang-2 expression.

5 produced Ang-2 mRNA. Two additional cancers had no detectable HER2 expression by ELISA yet produced Ang-2 mRNA. Further, 1 breast cancer cell line, MDA-MB-231 cells, expressed HER2 but had no detectable Ang-2 mRNA production. These data support the HER2 signaling link to Ang-2 production but clearly illustrate that HER2 expression and signaling are not the only potential pathways to induce Ang-2 mRNA. It is possible that other factors that alter cell growth independent of HER2 may induce Ang-2 expression.

Published data suggest that relative dimerization patterns of HER2 with other members of the type I growth factor receptor family can influence particular cell signaling pathways stimulated by HER2 signaling.²⁰ This compartmentalization of signaling can support a variety of transcription pathways. Using ovarian cell lines, Xu et al²¹ showed that the level of HER2 expression, relative to HER3 and HER4, could modulate the response to heregulin, thereby determining whether a response is stimulatory or inhibitory. It is possible that other dimer-

ization patterns of these type I receptors, perhaps involving EGFR, can also induce a signaling pathway leading to Ang-2 production as seen in 2 of the breast cancer specimens. It is also possible that the compartmentalization of the signaling in the MDA-MB-231 cells bypasses Ang-2 mRNA transcription. Alternatively, the HER2 receptor expressed in these cells may not have an intact signaling mechanism. DiGiovanna et al²³ showed that the expression of HER2 does not necessarily imply signaling. In that study, only 35% of HER2-overexpressing breast cancers actively signaled through the HER2 receptor. Further delineation of the relative expression of all these receptors and the signaling transduction pathways in Ang-2 producing breast cancers would help to understand this mechanistic pathway.

In this report, we have identified Ang-2 production in human breast cancer cells that express HER2 and further linked the regulation of Ang-2 production in these cells to HER2 signaling. We also identified Ang-2 mRNA in human breast cancer specimens and provided evidence of a link between HER2 expression and Ang-2 in these tumors. The findings of HER2 signaling-linked Ang-2 production makes Ang-2 and its receptor, Tie-2, potential therapeutic targets for the treatment of HER2 that expresses breast carcinoma, which may act synergistically with Herceptin. Further, the expression of Ang-2 may define which patients may benefit from Herceptin therapy.

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HER2 signaling–induced microvessel dismantling

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Background. The human epidermal growth factor receptor 2 protein (HER2) signaling in breast cancer imparts a metastatic advantage to the cell, likely by regulating gene expression. The HER2 signaling up-regulates angiopoietin-2 (Ang-2), which disrupts endothelial cell (EC) adherens junctions. We postulated that HER2 signaling may facilitate angiogenesis by disrupting microvessel integrity.

Methods. Rat microvessels, embedded in collagen, were grown into capillary networks and cocultured with MCF-7 or HER2 overexpressing MCF-7 (HER) to test for microvessel breakdown. We quantitated this effect by determining the cumulative length of intact microvessels. Other experiments used Herceptin- or heregulin β 1-pretreated MCF-7 cells to modulate HER2 signaling, or soluble Tie-2/Fc receptor fusion protein (sTie2) to sequester tumor-cell released Ang-2.

Results. The MCF-7 cells induced a time-dependent loss of microvessel integrity. At 12 hours, HER cells induced a 90% reduction in cumulative length ($P < .05$). Pretreatment with Herceptin reduced whereas heregulin β 1 augmented microvessel dismantling ($P < .01$). Sequestration of Ang-2 significantly, though not dramatically, reduced the MCF-7 cell induction of microvessel dismantling ($P < .01$).

Conclusions. We show that HER2 signaling in breast cancer cells leads to induction of microvessel dismantling, which may open a portal for angiogenesis. It appears that Ang-2 affects this mechanism, although other factors also function in microvessel dismantling. (Surgery 2001;130:382-7.)

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THE OVEREXPRESSION OF HER2 (neu, c-erbB2) imparts an aggressive metastatic phenotype in breast cancer. Second only to lymph node status, HER2 protein overexpression is a powerful predictor of whether the patient is disease free and of the survival prognosis.¹⁻³ A member of the epidermal growth factor receptor family, HER2 is expressed in a range of cell types and functions in growth, differentiation, and survival.^{4,5} The ligand class for these receptors is the neuregulin, which is relatively specific in its binding. Neuregulin binding induces heterodimerization with HER2, which is the signaling subunit and has no independent ligand.^{6,7} The relative dimerization patterns of HER2 with other receptors influences specific cell signaling pathways.⁸ This signaling compartmentalization can support a variety of transcription path-

ways, with divergent downstream events. The dimerization pattern of HER2 with HER3 relative to HER4 can modulate the response to heregulin, inducing a response that is either stimulatory or inhibitory.⁹ Antibody blockade of HER2, which prevents heterodimerization, can eliminate neuregulin-stimulated signaling.¹⁰⁻¹³ Signal down modulation can induce tumor regression in breast cancer cells expressing HER2.^{14,15}

The metastatic advantage imparted to cancer cells by over-expressing HER2 has not been clearly delineated. In testing the hypothesis that HER2 signaling is linked to cellular production of factors that influences the metastatic phenotype, we have shown that breast cancer cells expressing HER2 can induce endothelial cells (ECs) to retract, creating a gap in the endothelial monolayer.¹⁶ This gap may serve as a portal for tumor cell transendothelial migration, a key step in the metastatic process. This ability of the cell to induce EC retraction can be regulated by modulation of HER2 signaling by using monoclonal antibody blockade of HER2 dimerization or signal induction with heregulin β 1 stimulation. Oh et al¹⁷ also have identified downstream alterations in gene expression as a direct consequence of HER2 signaling.

Because of its profound effects on ECs, we have

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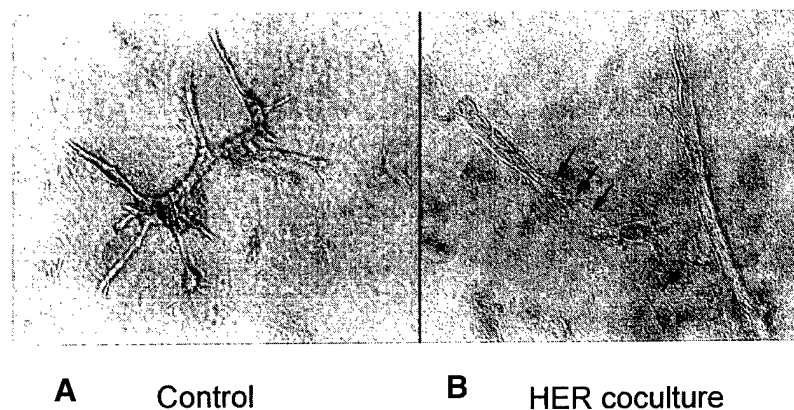


Fig 1. Dismantling of microvessel networks by coculture with HER cells. **(A)** Control vessels after 8 days in collagen I gel with DMEM + 10% FBS. **(B)** Microvessels after 12 hours of coculture with 1×10^3 HER cells/mm². Arrows indicate regions of dismantling and microvessel discontinuity. Images are phase contrast at 4 \times and embossed to enhance detail.

postulated that Ang-2 may be a factor involved in HER2-induced EC retraction. Ang-2 is a key protein involved in angiogenesis, the development of neovessels from existing blood vessels. Angiogenesis involves EC dissociation, shape change, and invasion of the supporting matrix.¹⁸ The EC specific tyrosine kinase receptors, Tie-1 and Tie-2 (tek), appear to be key signaling elements involved in angiogenesis. The ligands for Tie-2, angiopoietin-1 (Ang-1), and Ang-2 are antagonistic although they bind with equal affinity.¹⁹ The Ang-1 appears to function in the stabilization of vessels, whereas Ang-2 binding to Tie-2 results in destabilization of the vessel with dissociation of EC-EC junctions.

We have shown that HER2 signaling induces EC retraction. The mechanism appears to be the induction of dissociation of α , β , and γ catenin from VE cadherin, breaking the link between the adherens junction and the cytoskeleton.²⁰ We also have shown that HER2 signaling appears to regulate the cellular production of Ang-2 in MCF-7 cells,²¹ and we have linked Ang-2 production to EC retraction and the catenin:cadherin dissociation.

These findings led us to hypothesize that HER2 signaling-induced production of Ang-2 in breast cancer cells would initiate vascular disassembly, imitating the angiogenic regression phase. This architectural dismantling of microvessels potentially would open a portal for angioinvasion of these phenotypic cells, a key step in the metastatic process.

In this study, we tested MCF-7 cells and HER2 overexpressing MCF-7 cells for the ability to induce microvessel dismantling of an *in vitro* microvessel network. Further, we manipulated HER2 signaling by using Herceptin and heregulin β 1 to test for

HER2 regulation of microvessel dismantling. Finally, we treated MCF-7 cells with Tie-2/Fc soluble receptor fusion protein to quench cell-released Ang-2 and tested for microvessel dismantling.

MATERIAL AND METHODS

Cell lines. The MCF-7, SK-Br3, and MDA-MB-175 cells (American Type Culture Collection) and HER cells, a stable transfected MCF-7 cell line overexpressing HER2 6-fold (gift of C. Benz, San Francisco, Calif²²) were maintained in high glucose, Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (1000 U/mL) and streptomycin (1 mg/mL), at 37°C, 5% CO₂. The HER cells also were supplemented with G418 (300 μ g/mL) to maintain selection of expressing, transfected cells.

Microvessel dismantling assay. Epididymal fat pads were harvested from male 200-g Sprague-Dawley rats. Approximately 10 g of fat were minced and digested with a 0.25% collagenase III (Worthington Biochemical Corp, Lakewood, NJ) solution for 5 minutes. The tissue was rinsed in phosphate-buffered saline solution + 0.2% bovine serum albumin, and sequentially filtered by using mesh sizes of 350 μ m and 30 μ m to isolate microvessel fragments from undigested tissue and subsequently from cells and debris. Eight to 10,000 vessels/mL were suspended in a matrix of collagen I (3 mg/mL) in DMEM and incubated in humidified 37°C and 5% CO₂, with DMEM + 10% fetal bovine serum in 24-well tissue culture plates. Vessels remained in culture for 12 days to allow for capillary network development. Testing of cancer cell lines occurred on day 12. Cells were harvested from log-phase growth cultures and added to wells at 1×10^3

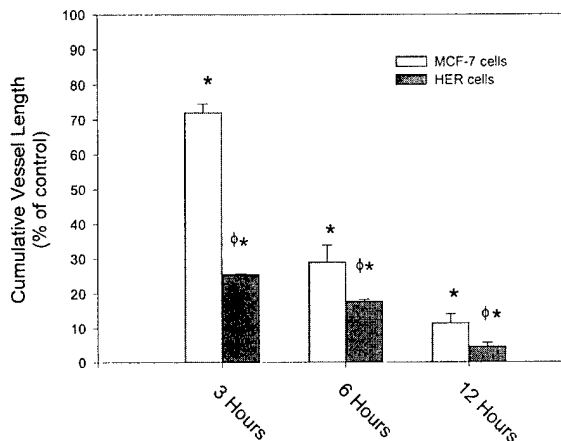


Fig 2. The HER2 induced microvessel dismantling. The MCF-7 or HER cells are cocultured at 1×10^3 cells/mm² with 12-day-old microvessels embedded in collagen I gel. Cumulative microvessel length was determined and compared with control (untreated microvessel gels.) The HER cells cause a more rapid and greater extent of dismantling compared with parental MCF-7 cells ($P < .05$ for all time points between cell types, and asterisk, $P < .01$ for both cell lines compared with control, $n = 12$ MCF-7; $n = 3$ HER).

cells/mm². For HER2 signaling experiments, MCF-7 cells were pretreated with 1 or 100 ng/mL Herceptin (gift of Genentech, San Francisco, Calif), or 0.1 or 1.0 ng/mL heregulin $\beta 1$ (Genentech) for 24 hours before the microvessel-dismantling assay. In other experiments, 2, 20, or 200 ng/mL of sTie2 was added with MCF-7 cells or alone to the microvessel cocultures. After 3 to 12 hours of coculture, the gels were fixed with 3.2% paraformaldehyde, and stained with rhodamine-conjugated Griffonia simplicifolia-1 (Sigma Chemical Co, St Louis, Mo), a lectin that specifically intercalates into rat endothelium. The gels then were sandwiched between glass slides and imaged by using an Olympus BH-2 microscope equipped with a Pixera digital camera (Pixera Corp). Microvessel length was calculated by linear tracing of the stained microvessels, minus the parent vessels, by using SigmaScan Pro image analysis software. Statistical analysis was performed by Student *t* test and analysis of variance between groups.

RESULTS

Rat microvessels in collagen I gels treated with MCF-7 cells experienced a structural integrity loss with disruption of length and development of isolated EC islands (Fig 1). The ECs appeared viable by trypan blue exclusion (data not shown). Because of the breakdown of architectural microvessel struc-

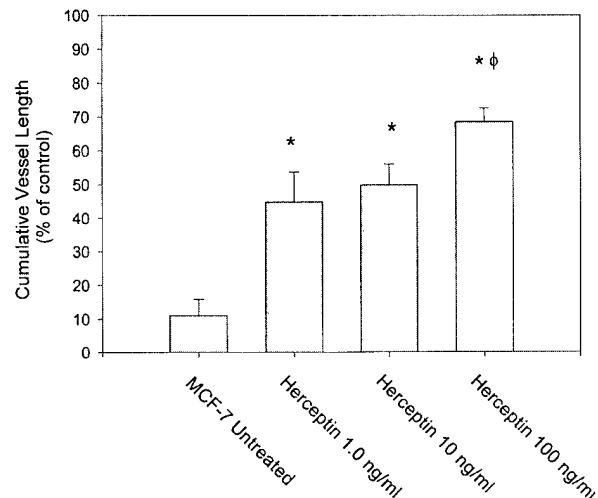


Fig 3. HER2 signaling blockade inhibited microvessel dismantling. MCF-7 cells were pretreated with increasing doses of Herceptin for 24 hours before coculture with microvessel collagen I gels for 12 hours. Cumulative microvessel length was determined and compared with control. Treated cells were dose-dependently inhibited from inducing dismantling (asterisk, $P < .01$ vs untreated MCF-7 cells; $P < .01$ vs 1.0 ng/mL; $n = 3$).

ture without loss of cellular integrity, we termed this phenomenon *microvessel dismantling*.

Because we have identified that HER2 signaling influences the induction of EC retraction in coculture with breast cancer cells expressing HER2, we compared MCF-7 cells with a MCF-7 derived cell line that overexpresses HER2 6-fold (HER cells) for the ability to induce microvessel dismantling in vitro. The MCF-7 treated cells showed a time-dependent decrease in cumulative length. At 3 hours, the treated microvessels retained only 72.3% \pm 2.1% of control length ($P < .01$), which was reduced to 11.5% \pm 2.6% of control by 12 hours ($P < .01$ vs 3 hours, Fig 2). HER cells induced a greater loss of microvessel integrity over time than the parental MCF-7 cells. At 3 hours, HER cell treated microvessel gels retained only 26% \pm 0.8% of control length ($P < .01$ vs MCF-7), which was reduced to 4.6% \pm 1.1% by 12 hours ($P < .01$ vs MCF-7, Fig 2).

To further evaluate HER2 signaling influence on microvessel dismantling, we pretreated MCF-7 cells with increasing doses of Herceptin for 24 hours before the microvessel dismantling assay. A dose-dependent blockade of MCF-7 induced microvessel dismantling was seen at 12 hours (Fig 3). Herceptin treatment (1.0 ng/mL) of MCF-7 cells reduced dismantling to 46.3% \pm 7.1% of control ($P < .05$ vs untreated MCF-7). Increasing doses of Herceptin further reduced dismantling, reaching

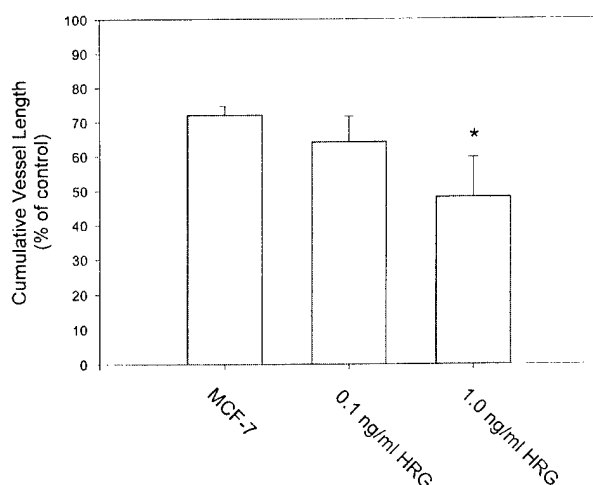


Fig 4. Stimulation of HER2 signaling augmented microvessel dismantling. The MCF-7 cells were pretreated with 0.1 or 1.0 ng/mL heregulin β 1 for 24 hours before coculture with microvessel collagen I gels for 3 hours. Cumulative microvessel length was determined and compared with control. Treated cells were dose-dependently enhanced in inducing dismantling, reaching significance at 1.0 ng/mL (* $P < .01$ vs untreated MCF-7 cells; $n = 3$)

68.6% \pm 3.9% of control with 100 ng/mL ($P < 0.01$ vs untreated MCF-7).

Stimulation of HER2 signaling in MCF-7 cells using heregulin β 1, resulted in accelerated microvessel dismantling (Fig 4). Cumulative length of intact microvessels at 3 hours was reduced to 48.6% \pm 11.7% of control ($P < .01$) from 72.3% \pm 2.1% after pretreatment with 1.0 ng/mL heregulin β 1 for 24 hours before the microvessel-dismantling assay.

To determine whether other HER2 overexpressing breast cancer cell lines also could induce microvessel dismantling, SK-Br3 and MDA-MB-175 cells were cocultured for 12 hours with microvessel gels. Both cell lines induced microvessel dismantling with significant reduction in cumulative length of intact microvessels compared to untreated controls ($P < .01$, Fig 5). Pretreatment with 1.0 or 100 ng/mL of Herceptin for 24 hours reduced microvessel dismantling in MDA-MB-175 cells ($P < .01$ vs untreated cells). Pretreatment of SK-Br3 cells with Herceptin did not significantly reduce cumulative microvessel length at 12 hours of coculture.

The HER2 signaling has been shown to regulate Ang-2 production. Because of the profound effect of Ang-2 in inducing angiogenesis, we pretreated MCF-7 cells with sTie2 to sequester any Ang-2 protein released. By sequestering Ang-2, we attempted to implicate Ang-2 as a participant in the microvessel dismantling mechanism. The MCF-7 cells were pretreated for 24 hours with increasing

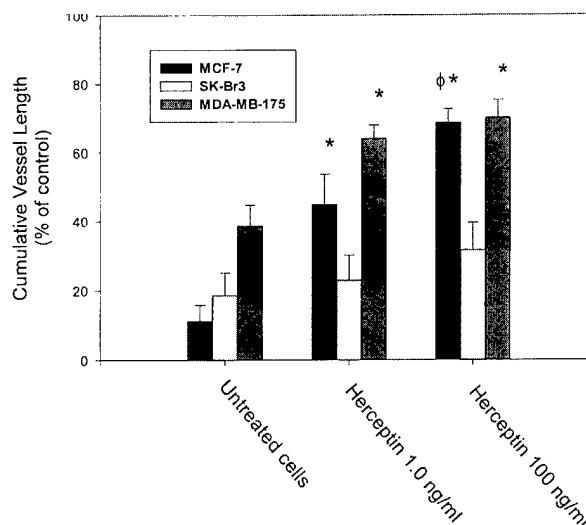


Fig 5. HER2 signaling induced microvessel dismantling in HER2 overexpressing cell lines. Untreated cells or Herceptin pretreated cells for 24 hours were placed in coculture with microvessel gels for 12 hours. Cumulative length of microvessels was determined and compared with control. All cell lines induced microvessel dismantling. HER2 signaling blockade with Herceptin inhibited induced dismantling in MCF-7 and MDA-MB-175 cells (* $P < .01$ vs untreated cells) but not SK-Br3 cells ($P =$ not significant; $n = 3$).

doses of sTie2 before coculture in the dismantling assay. Sequestration of Ang-2 with sTie2 significantly though not dramatically reduced MCF-7 induced microvessel dismantling (Fig 6). At 200 ng/mL, cumulative length of microvessels cocultured with sTie2/Fc treated MCF-7 cells was 24.5% \pm 5.5% of control ($P < .01$ vs untreated MCF-7 and MCF-7 treated with 2 ng/mL sTie2).

DISCUSSION

The HER2 overexpression induces a more aggressive phenotype in breast cancer cells. Early metastasis, earlier relapse, and a poorer overall prognosis accompany HER2 overexpression.¹⁻³ The finding that overexpression of a tyrosine kinase receptor induces a more aggressive phenotype empirically suggests that signaling leads to significant downstream gene modulation with gene product alteration. This gene product alteration likely must influence the local cancer environment, either directly or indirectly, to favor the more aggressive phenotype. In support of this hypothesis, Oh et al¹⁷ identified several genes with expression levels in MCF-7 cells that were significantly altered by HER2 overexpression induced by transfection. Presumably, monoclonal antibody blockade of HER2 dimerization and signaling should

eliminate the selection of downstream gene expression, which favors the aggressive phenotype. Indeed, Herceptin treatment appears to lengthen remission episodes and delay return to an aggressive phenotype while rendering the cell static.^{14,15}

Our prior studies have demonstrated that HER2 signaling regulates the induction of EC retraction with loss of cell-cell contact and the endothelial monolayer barrier.¹⁶ The EC retraction creates a portal for transmigration across the endothelium and potential egress into a secondary tumor site. This process appears to be induced by secreted product or products regulated by HER2 signaling. In this present study, we have further characterized the vascular modulation induced by HER2 signaling. The HER2 overexpressing breast cancer cells may enhance metastatic capability by inducing an architectural disassembly of intact microvessels, creating a noncontiguous capillary network. The microvessels appeared to have disrupted EC contact with loss of continuity, a phenomenon we termed *dismantling*. This break in continuity suggests the possibility of tumor cell access into the vascular system and possible earlier disseminated metastasis.

We show that microvessel dismantling is at least partially regulated by HER2 signaling. Blockade of HER2 signaling resulted in a dose-dependent decrease in the ability to induce microvessel dismantling. Conversely, stimulation of HER2 signaling increased the cells' influence on microvessel integrity.

The process was not exclusive to MCF-7 cells. Both MDA-MB-175 cells and SK-Br3 cells also induced dismantling. The MDA-MB-175 cells were regulated similarly in this induction, shown by blockade of HER2 signaling with reduction in dismantling potential. The SK-Br3 cells were not influenced significantly by Herceptin blockade. It is possible that SK-Br3 cells process alternate or defective signaling or possibly influence different downstream gene transcription with different phenotypic expression. Xu et al⁹ showed that the relative heterodimer expression could substantially change downstream phenotypic expression. By exogenously influencing relative levels of expression of HER3 to HER4 heterodimers with HER2, phenotypic expression shifted from a stimulatory pattern to an inhibitory pattern.

Because our prior studies have shown that HER2 signaling can regulate Ang-2 expression and further that Ang-2 appears to be involved in the mechanism of EC retraction induced by HER2 signaling, we postulated that Ang-2 may be the key factor involved in the induction of microvessel dismantling.

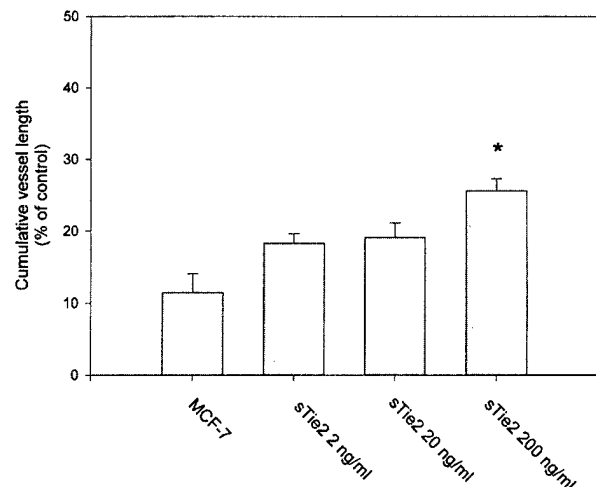


Fig 6. sTie2/Fc sequestration of MCF-7 produced Ang-2 inhibits microvessel dismantling. MCF-7 cells were treated with increasing doses of sTie2/Fc and placed in coculture with microvessel collagen I gels for 12 hours. Cumulative length of microvessels was determined and compared with control. sTie2/Fc dose-dependently inhibited induced dismantling, reaching significance at 200 ng/mL (* $P < .01$ vs control, 2, and 20 ng/mL; $n = 3$).

ting. Using sTie2/Fc to sequester Ang-2 released from MCF-7 cells, we showed a significant though not dramatic reduction in the loss of microvessel integrity. Unfortunately at this time, an adequate Ang-2 protein was not available to test directly. A crude preparation containing some intact Ang-2 protein was tested, but no significant induction of microvessel dismantling was identified (data not shown.) It appears that Ang-2 is likely a factor influencing the mechanism of dismantling, but is not the only or possibly even a major determinant of the process.

Microvessel dismantling also may be involved in an angiogenic response induced by HER2 signaling regulation of Ang-2 and other EC mitogenic factors. The Ang-2 expression in tumors occurs at sites of angioregression and capillary breakdown.²³ It appears that in angiogenesis, Ang-2 may influence EC-EC dissociation and release of anchoring, allowing mitogenic stimulation for proliferation and migration. The observed microvessel dismantling in this study essentially may be part of tumor co-option of microvessels with release of ECs, angioregression, and ultimately tumor induced angiogenesis.²⁴ In this scenario, discontinuity and possible angioinvasion may be a mere byproduct of tumor and environmental factors inducing an angiogenic response. The Ang-1 knockout mice, providing unopposed Ang-2 influence on Tie2 receptors, were noted to have discontinuous vascu-

lar networks with islands of isolated EC, offering some support for a role for Ang-2 in the dismantling mechanism.¹⁹ Other factors such as urokinase plasminogen activator, a proteolytic enzyme, may influence both angiogenesis and tumor neovascularization in breast cancer by vessel wall degradation, providing a possible portal for tumor dissemination or release of EC for angiogenesis.²⁵

In conclusion, we have demonstrated a phenotypic expression of HER2 signaling in MCF-7 breast cancer cells, induction of microvessel dismantling, which is likely stimulated by downstream expression of gene products favoring a metastatic profile. This process appears to be common to other HER2 overexpressing cell lines. The mechanism appears to involve cellular production of Ang-2, but clearly other factors that influence vascular structures are active in the mechanism. This study suggests that HER2 signaling induced metastatic pathways likely involve opportunistic expression of angioactive factors, which may provide a portal for angiogenesis.

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HER2 OVEREXPRESSION ENHANCES TUMOR CELL TRANSENDOTHELIAL MIGRATION

WB Carter*, JB Hoying, SK Williams, Division of Surgical
Oncology, Univ. of Maryland, Baltimore, MD.

Overexpression of HER2 in breast carcinoma imparts a metastatic advantage. Metastasis requires escape of the tumor cell from the vasculature into subjacent tissue, a transmigration event across an endothelial cell (EC) monolayer. We have shown that HER2 signaling induces EC retraction and loss of the intact endothelial barrier.

Using a novel in vitro assay, we tested the ability of MCF-7 cells and stable, transfected MCF-7 cells which overexpress HER2 6 fold (HER cells) to transmigrate across intact human EC monolayers. We further tested HER2 signaling blockade (Herceptin™) to determine if HER2 signaling regulates transmigration. MCF-7 or HER cells were cocultured onto mature EC monolayers. Using total internal reflection fluorescence (TIRF) microscopy, we determined cell transmigration efficiency. After treatment with Herceptin™ for 24 hrs, the MCF-7 cells were further tested for transmigration.

By 3 hrs, the transmigration efficiency of HER cells was $74 \pm 4.7\%$ vs $42 \pm 6.9\%$ for MCF-7 cells ($p < 0.01$). Using modified Boyden chambers, no differences were found in cell motility or matrix invasion between the cell groups. Blockade of HER2 signaling with Herceptin™ (200 ng/ml) reduced transmigration efficiency by 33% ($p < 0.05$).

These data suggest that HER2 signaling may enhance the metastatic phenotype in breast cancer by improving the efficiency of tumor cell transmigration across EC. Further, HER2 signaling blockade may limit tumor spread by delaying tumor cell escape from the vasculature.

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